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IspG Converts an Epoxide Substrate Analogue to (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate: Implications for IspG Catalysis in Isoprenoid Biosynthesis

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Abstract

Isoprenoids comprise a vast and structurally diverse class of natural products that are assembled from two simple precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Essential for all living organisms, IPP and DMAPP are biosynthesized by two biosynthetic pathways. Animals, fungi, and archae bacteria utilize the mevalonate pathway for production of IPP and DMAPP,[1] whereas bacteria, parasites, and plants rely upon the methylerythritol phosphate (MEP) pathway (Scheme 1) for biogenesis of these two central precursors.[2–8] Its essentiality and prevalence in plants and human pathogens renders the MEP pathway an attractive new target for the development of herbicides and anti-infective agents.

Among the most mechanistically intriguing transformations of the seven-step MEP pathway is the penultimate step catalyzed by IspG (Scheme 1). IspG utilizes a cyclic diphosphate, methylerythritol cyclic diphosphate (MEcPP, **6**), as substrate for a reductive ring-opening reaction to form (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP, **7**). Recently, we greatly enhanced the IspG enzymatic activity relative to that reported in previous work.[9] With the use of dithionite as the reductant in combination with a proper mediator to facilitate the reduction of the IspG Fe–S cluster, the IspG activity was increased by ~20-fold relative to that of the NADPH–flavodoxin reductase–flavodoxin reducing system.[6] This dramatic improvement in IspG activity permits more detailed studies to elucidate the IspG catalytic mechanism.

Several mechanisms have been proposed for IspG.[8,10–12] A particularly intriguing and frequently cited mechanism was proposed by Rohdich et al.[11] and involves the C2-hydroxyl-assisted ring opening of MEcPP (Scheme 2) to generate an epoxy intermediate, (2*R*,3*R*)-4-hydroxy-3-methyl-2,3-epoxybutanyl diphosphate (Epoxy-HMBPP, **10)**. The epoxide intermediate **10** is then converted to HMBPP via a reductive dehydration process. While the deoxygenation of epoxides to the corresponding olefins by a synthetic $[4Fe–4S]^{2+}$ cluster

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Supporting Information Available: Procedures for the synthesis of compound **10**, characterization of the IspG-catalyzed enzymatic product of **10**, and optimization of IspG activity for compound **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

has been demonstrated[13] and provides chemical precedence for such a mechanism, there is no evidence to date that IspG can convert the proposed expoxide intermediate **10** to HMBPP (**7**). Here we report the synthesis and characterization of **10** and demonstrate that it is catalytically competent as a substrate for IspG in the formation of HMBPP.

Compound **10** was synthesized as shown in Scheme 3. Benzyl ether **15** was prepared from dimethylallyl alcohol in 52% yield over five steps. Stereoselective incorporation of the epoxide moiety into compound **16** was accomplished using Sharpless epoxidation conditions.[14] Mesylation of **16** and subsequent debenzylation using transfer hydrogenolysis afforded mesylate **18** in 67% yield over two steps. Coupling of tris(tetrabutylammonium) diphosphate to **18** was accomplished using the general procedure reported by Davisson et al.[15] and afforded epoxide analogue **10** in 40% yield.

Compound **10** was evaluated as an IspG substrate using our recently reported ^{1}H NMR assay.[9] This assay monitors IspG activity by detecting the C3′ methyl protons of MEcPP (1.26 ppm) and HMBPP (1.54 ppm) (see the Supporting Information). The C3′ methyl proton of epoxide analogue **10** has a chemical shift of 1.22 ppm (Figure 1a, trace A). As shown in Figure 1, IspG catalyzes the conversion of **10** to HMBPP (**7**) in an enzyme-dependent manner (Figure 1a, traces B–E). The enzyme-generated product was purified by HPLC and characterized using ^{1}H NMR and high-resolution mass spectrometry. The data are consistent with the production of HMBPP as the product (see the Supporting Information).

We further characterized the epoxide analogue **10** by kinetic analysis (Figure 1c) using dithionite as the reducing agent and methyl viologen (MV) as the redox mediator.[9] Initial velocities were measured by monitoring the consumption of reduced MV at 734 nm.[9] As summarized in Table 1 and Figure 1c, 10 is a kinetically competent IspG substrate exhibiting a k_{cat} of 20.1 min $^{-1}$, which is very close to that of the natural IspG substrate MEcPP (**6**) (Figure 1b, Table 1). Interestingly, the K_m for **10** (K_m = 119 ± 24.5 μM) is ~3-fold smaller than that of **6** (K_m = 311 ± 21.4 μM), and apparent substrate inhibition was observed (K_i = 1.3 ± 0.4 mM).

Our studies indicate that **10**, an intermediate in the IspG reaction mechanism proposed by Rohdich et al.,[11] is catalytically competent as an IspG substrate, and they provide the first direct experimental evidence suggesting that an epoxide intermediate is possible in the catalytic mechanism of this intriguing enzyme. The kinetic parameters of **10** relative to MEcPP suggest the possibility that IspG could accommodate linear diphosphate **10** with an affinity comparable to that for the structurally dissimilar MEcPP. The lower K_m value of **10** is consistent with an enzymatic conformational state along the natural reaction pathway that has high affinity for the linear diphosphate epoxide intermediate, to prevent nonproductive release into solution. Furthermore, the similar k_{cat} values suggest that the natural substrate and the epoxide share a common rate-limiting step that is post-epoxide formation, if the epoxide is indeed an intermediate along the reaction pathway.

Supplementary Material

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Acknowledgments

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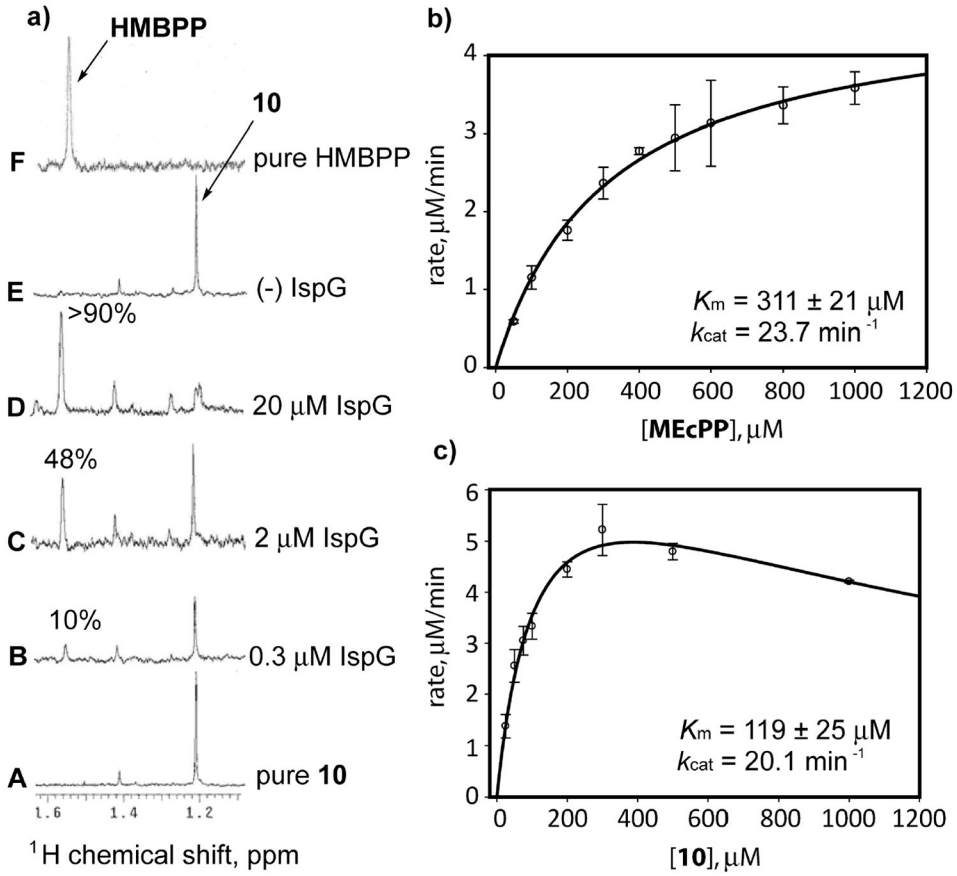
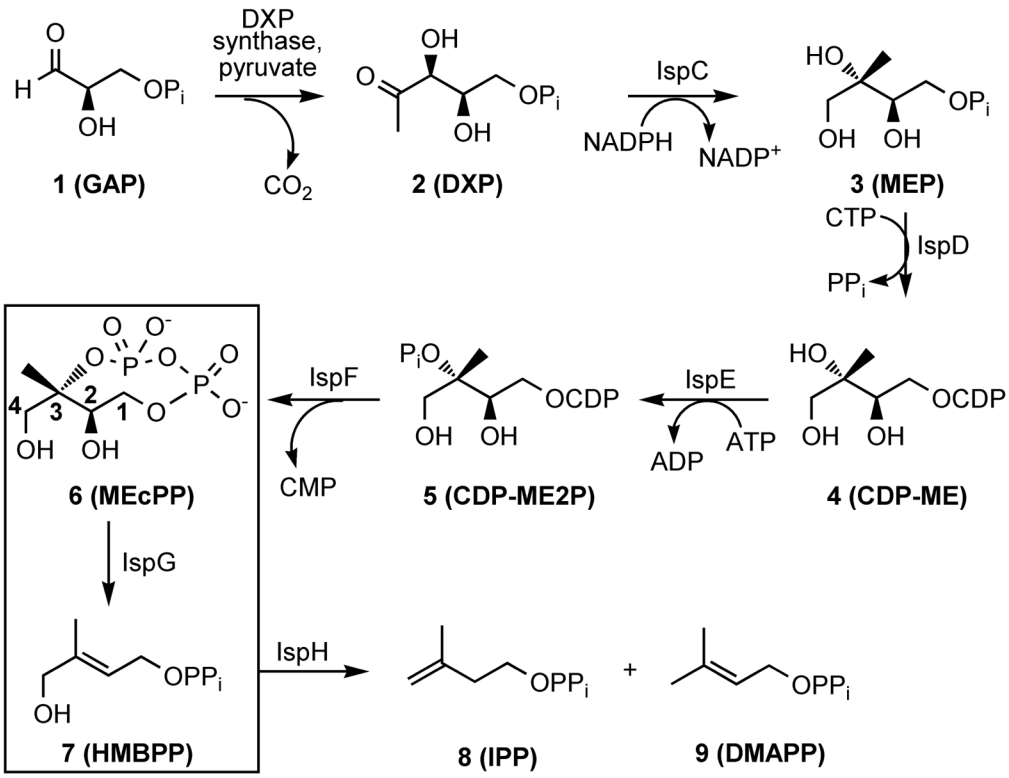


Figure 1.

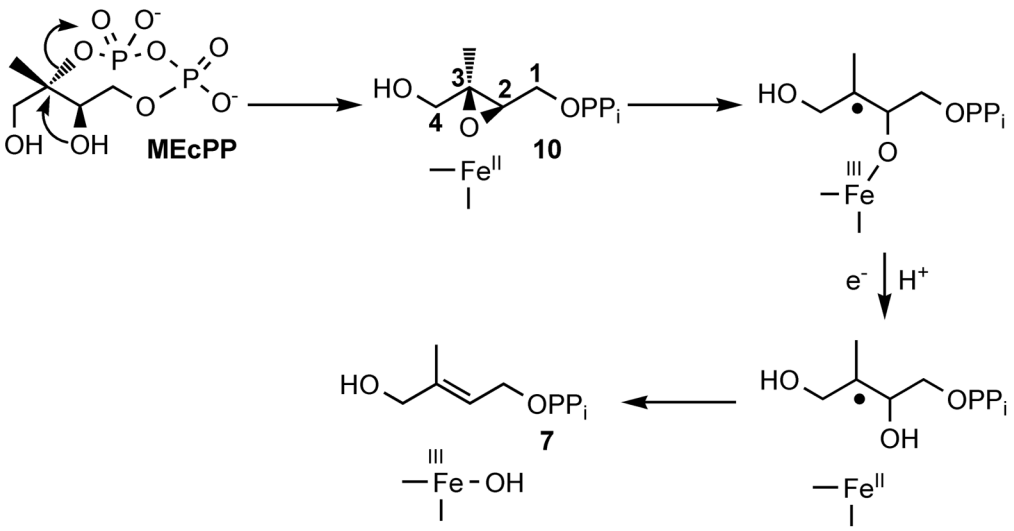
(a) ^{1}H NMR analysis of the IspG-catalyzed conversion of **10** to HMBPP. Reaction conditions: 100 mM Tris buffer (pH 8.0) at 37 °C for 30 min with 5 mM dithionite, 1 mM MV, 1 mM **10**, and various IspG concentrations. (b) Michaelis–Menten analysis of MEcPP (**6**). (c) Michaelis–Menten analysis of **10**.



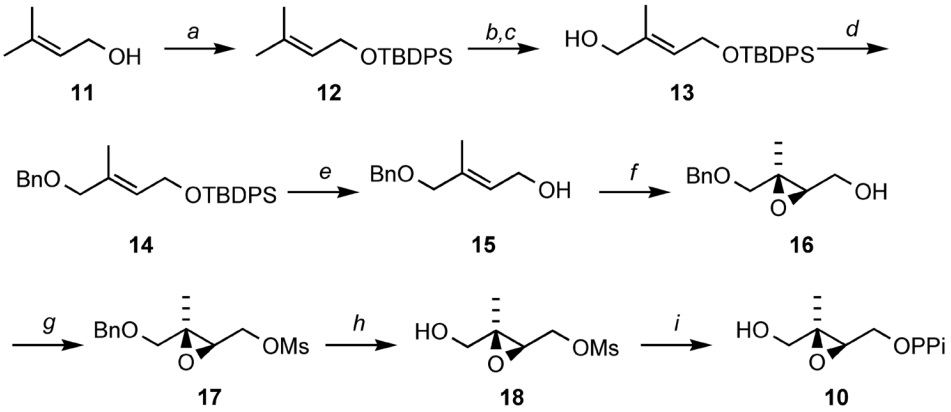
Scheme 1.

Methylerythritol Phosphate (MEP) Pathway[a]

[a] Abbreviations: GAP, glyceraldehyde phosphate; DXP, deoxyxylulose phosphate; CDP-ME2P, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; CDP-ME, methylerythritol cytidinyl diphosphate; MEcPP, methylerythritol cyclic diphosphate; HMBPP, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate.



Scheme 2.
IspG Mechanistic Model Proposed by Rohdich et al.[11]



Scheme 3.

Synthesis of Epoxide Analogue **10**[a]

[a] Conditions: (a) Imidazole (2.5 equiv), TBDPSiCl (1.1 equiv), 5:1 DMF/CH_2Cl_2, 0 °C to rt, 15 h, 100%. (b) SeO_2 (0.5 equiv), *t*-BuOOH, CH_2Cl_2, 0 °C to rt, 4 h. (c) $NaBH_4$, MeOH, 0 °C, 5 min, 64% over two steps. (d) NaH, BnBr, THF, 0 °C to rt, 15 h, 95%. (e) TBAF, THF, rt, 30 min, 86%. (f) (−)-D-DET (2 equiv), $Ti(OiPr)_4$ (1.5 equiv), *t*-BuOOH (3 equiv), CH_2Cl_2, −25 to −20 °C, 14 h, 63%. (g) MsCl, Et_3N, CH_2Cl_2, −78 °C, 30 min, 98%. (h) Pd/C, 1:24 HCO_2H/MeOH, rt, 1–12 h, 76%. (i) $P_2O_7H \cdot 3NBu_4$, CH_3CN, rt, 30 min, 40%.

Table 1

Kinetic Parameters for **10** and MEcPP (**6**)

substrate	IspG (nM)	K_m (μM)[a]	V_{max} (μM/min)	k_{cat} (min^{-1})	K_i (mM)
MEcPP	200	311 ± 21	4.7 ± 0.1	23.7	n.d.
10	400	119 ± 25	8.0 ± 0.9	20.1	1.3 ± 0.4

[a]The following equations were used for MEcPP and **10**, respectively: $v = V_{max}[S]/(K_m + [S])$ and $v = V_{max}/(1 + K_m/[S] + [S]/K_i)$.